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Amplification of *Chlamydia trachomatis* DNA by Ligase Chain Reaction

BRUCE J. DILLE,* CARLA C. BUTZEN, AND LARRY G. BIRKENMEYER

Experimental Biology Research, Abbott Diagnostics Division, North Chicago, Illinois 60064

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Amplification of *Chlamydia trachomatis* DNA by polymerase chain reaction was compared with amplification by ligase chain reaction (LCR). Both amplification procedures were able to consistently amplify amounts of DNA equivalent to three *C. trachomatis* elementary bodies. All 15 *C. trachomatis* serovars were amplified to detectable levels by LCR, and no DNA from 16 organisms potentially found in clinical specimens or from *Chlamydia psittaci* and *Chlamydia pneumoniae* was amplified by LCR.

Chlamydia trachomatis is an obligate intracellular parasite which has 15 serovars distinguished by variable regions of the major outer membrane protein (MOMP) and contains a 7.5-kb cryptic plasmid which appears to be required for intracellular growth (13, 27). *C. trachomatis* is a major human pathogen causing a variety of diseases. There are approximately 4 million cases of *C. trachomatis*-caused sexually transmitted disease occurring annually in the United States alone (14, 16, 17). Exposure to *C. trachomatis* during birth can lead to conjunctivitis and pneumonia in the neonate (1). *C. trachomatis* also causes trachoma, the leading cause of preventable blindness worldwide. Over 500 million people are estimated to be infected, with 7 million being blind (6).

No diagnostic method for *C. trachomatis* has yet been shown to approach 100% sensitivity (2). The "gold standard" with which all other methods are compared is cell culture, which detects at best 80% of genital infections and less than 50% of trachoma and the results of which can vary considerably depending on sample handling and culture procedures (12, 21). Nonculture methods have been developed to lessen the complexity and decrease the time required for chlamydia detection (5, 12, 22–25). Recently, polymerase chain reaction (PCR) procedures have been used to amplify chlamydia-specific DNA sequences prior to detection to increase sensitivity (7, 8, 18, 19).

In this study amplification of *C. trachomatis* DNA by PCR was compared with amplification by ligase chain reaction (LCR). A detailed description of LCR has been published in a recent review (4). Briefly, LCR uses two complementary pairs of probes which, when the correct template is available, hybridize next to each other and then are ligated together. These ligated probes plus the original template serve as the template for the next cycle of hybridization and ligation. As subsequent cycles are performed, the amplification proceeds exponentially (4, 9). LCR probe sets were evaluated for amplification of all 15 serovars of *C. trachomatis* and a panel of organisms potentially found in clinical specimens plus *Chlamydia psittaci* and *Chlamydia pneumoniae*.

Comparison of PCR and LCR. A dilution panel of *C. trachomatis* serovar L2 was prepared from stocks grown in McCoy cells, and elementary bodies (EB) were counted by light microscopy with Giemsa stain. The DNA was extracted by proteinase K digestion followed by chloroform-phenol extraction and ethanol precipitation (20). Both amplification procedures were able to amplify and consistently detect three EB with all primers and probes tested.

For the PCR, 5 μl of sample was used in a 100-μl mixture consisting of 10 μl of 10× PCR buffer (Perkin-Elmer Cetus, Norwalk, Conn.), 200 μM deoxynucleotide triphosphates (Pharmacia, Uppsala, Sweden), 1 μM each DNA primer (MOMP, 5′-GCCGCTTTGAGTTCTGCTTCCTC-3′ and 5′-CCAAGTGGTGCAAGGATCGCA-3′) (7), and 2.5 U of thermostable polymerase (AmpliTaq; Perkin-Elmer Cetus). The mixture was overlaid with oil and placed in a thermocycler (Coy Laboratory Instruments, Ann Arbor, Mich.). Samples were cycled 29 times at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min each time. A final cycle had the same 94 and 55°C steps but was held at 72°C for 10 min.

After amplification 33 μl was removed from the tube and mixed with 117 μl of TE (10 mM Tris, 1 mM EDTA, pH 8) and 2.5 ng of human placental DNA (Sigma, St. Louis, Mo.) per ml, and then 15 μl of 4 N NaOH was added. The mixture was incubated at 65°C for 50 min, and then 165 μl of 2 M ammonium acetate was added. Half of the sample was put into a slot (Bio-Dot SF microfiltration apparatus; Bio-Rad, Richmond, Calif.) and blotted onto GeneScreen Plus (NEN Research Products, Boston, Mass.). The DNA was cross-linked to the membrane by UV (Stratalinker UV cross-linker; Stratagene, La Jolla, Calif.).

The membrane was blocked with a mixture containing 6× SSC (20× SSC is 3 M NaCl plus 0.3 M $Na_3C_6H_5O_7$, pH 8), 5× Denhardt's solution (50× Denhardt's solution is 5 g of Ficoll type 400, 5 g of polyvinylpyrrolidone, and 5 g of bovine serum albumin (BSA) fraction V per ml in 500 ml of H_2O), 0.1% sodium dodecyl sulfate (SDS), and 0.1 mg of salmon sperm DNA per ml for 3 h at 60°C. Hybridization was in the same buffer overnight at 60°C with radiolabeled probe added at 10^6 dpm/ml. The probe (5′-TCCTTGC AAGCTCTGCCTGTG-3′) (7) was radiolabeled with [^{32}P] ATP (3,000 Ci/mmol; Amersham, Arlington Heights, Ill.) by using T4 kinase following standard protocols (20). The blot was then washed with 2× SSC–0.5% SDS for 5 min at 22°C, 2× SSC–0.1% SDS for 15 min at 22°C, and twice in 2× SSC–0.5% SDS for 15 min each at 60°C. The dried membrane was placed on X-OMAT AR film (Kodak, Rochester, N.Y.) with intensifying screens (Lightning Plus; DuPont, Wilmington, Del.) to detect the radiolabeled slots. Figure 1a shows an autoradiograph of the slot blot. In this example chlamydia DNA was amplified and detected at an equivalent

* Corresponding author.

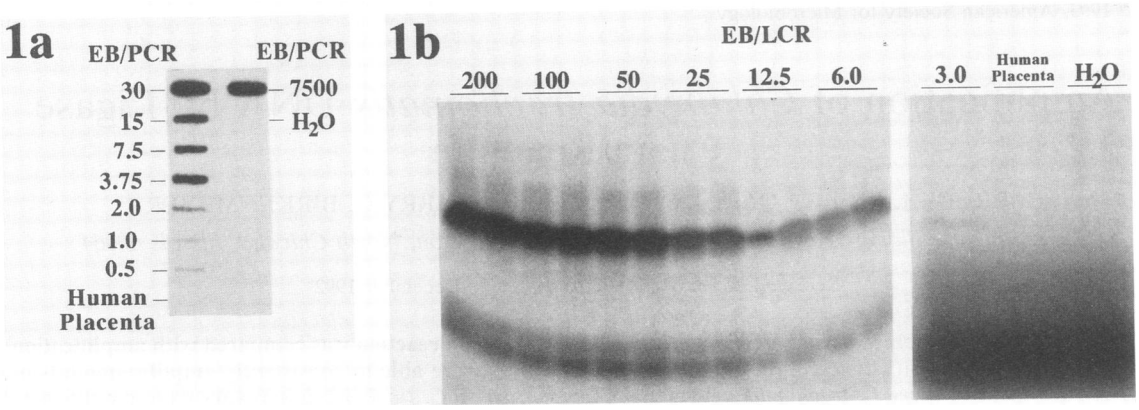

FIG. 1. Amplification of dilution panel by PCR and LCR. Serovar L2 was diluted, and DNA was extracted for either PCR or LCR amplification. The number of amplification cycles for each procedure was optimized to maximize specific sequence amplification without detecting any background signals for the detection method used. (a) Autoradiograph of slot blot of amplified product from PCR. After 30 cycles of amplification, sample was blotted onto GeneScreen Plus and hybridized with ^{32}P-labeled probe. The autoradiograph was developed after a 6-h exposure with two intensifying screens. (b) Autoradiograph of acrylamide gels of amplified product from LCR. LCR was carried out for 60 cycles with [^{32}P]dCTP incorporated directly into ligated probes. After cycling, half of the reaction mixture was electrophoresed on 10% polyacrylamide gels. Wet gels (no screens) were exposed for 2.5 h for 200 to 6.25 EB per LCR and 4 h for 3.13 EB per LCR, human placenta DNA, and H_2O. The lower bands were individual probes with [^{32}P]dCTP incorporated but not ligated.

of less than 0.5 genome per amplification reaction. This level of detection, however, was not consistently achieved because of the lower probability of pipetting one EB into the reaction mixture. Consistent amplification to detectable levels was seen at the three-EB dilution point.

For the LCR 5 μl of sample was also used, but the total reaction volume was 50 μl. The reaction mixture contained 8 μl of 5× LCR buffer {250 mM EPPS [*N*-(2-hydroxyethyl) piperazine-*N*-(3-propanesulfonic acid)], 50 mM $MgCl_2$, 50 mM NH_4Cl, 5 mM dithiothreitol, 50 μg of BSA, and 400 mM K^+ (as KOH and KCl), pH 7.8}, 0.2 μl of [^{32}P]dCTP or [^{32}P]dGTP (depending on the nucleotide needed to fill the gap in the particular probe set) (400 Ci/mmol; Amersham), 0.5 μl of 10 mM NAD^+, 6.25 × 10^{11} molecules of each probe, and 6 μl of enzyme mixture (2.5 μl of thermostable DNA ligase, 0.5 U of thermostable DNA polymerase [Ampli Taq; Perkin-Elmer Cetus], 1× LCR buffer to 6 μl). The ligase purified from *Thermus thermophilus* was kindly provided by R. Marshall (Abbott Laboratories). The probe sets were as follows: MOMP set 1 (EMBL accession number J03813, map numbers 36 to 89), 5′-TTTTACTTGCAAGA CATTCCTCAGG ATTAATTGCTACAGGACATCTTG TC-3′ and 3′-CGAAAATGAACGTTCTGTAAGGAGT GG TAATTAACGATGTCCTGTAGAAC-5′; MOMP set 2 (map numbers 552 to 595), 5′-GGGAATCCTGCTGAACCAAG TTATGATCGACGGAATTCTGTG-3′ and 3′-CCCTTAGG ACGACTTGGTTGGAATACTAGCTGCCTTAAGACAC-5′; and plasmid (map numbers 6693 to 6739 [10]), 5′-GATAC TTCGCATCATGTGTTCC AGTTTCTTTGTCCTCCTATA ACG-3′ and 3′-CTATGAAGCGTAGTACACAA CCTC AAAGAAACAGGAGGATATTGC-5′. The reaction mixture was overlaid with oil and placed in a thermocycler for 60 cycles of 85°C for 30 s and 50°C for 20 s. Half of the mixture was then electrophoresed in a 10% polyacrylamide gel (20), and film was exposed to the wet gels to detect product.

An autoradiograph of the acrylamide gel of the LCR product using MOMP probe set 1 revealed detection of amplification product from the three-EB dilution panel member (Fig. 1b). As with the PCR amplification, consistent amplification was not observed in dilutions below three EB. Similar results were observed for LCR amplification and detection of product with the other MOMP probe set and the plasmid probe set (data not shown).

The possibility of being able to detect as few as three EB in a clinical sample raises the question of the clinical significance of finding such low numbers in a patient. In one study determining inclusion-forming units (IFU) recovered from 1,231 infected women, 25% had fewer than 100 IFU/ml (3). In another study of 580 women, 34% had fewer than 100 IFU per monolayer (11). The number of EB per IFU can be influenced by a number of factors but will be an important consideration if the number of EB per IFU is high, resulting in apparent false-positive results. The extent and significance of these culture-negative, yet true-positive, samples will need to be investigated.

Serovar amplification and detection by LCR. Further evaluation of the LCR probes was done to assess their ability to amplify the 15 *C. trachomatis* serovars (American Type Culture Collection, Rockville, Md.). Extracted DNA from each serovar was diluted to an equivalent of 200 and 10 EB per amplification reaction and then amplified by LCR. All of the LCR probe sets were able to amplify to detectable levels all 15 serovars at 10 EB per reaction. In each set of amplification reactions, tubes containing either water (no DNA) or human placenta DNA were both included to control for potential cross-contamination.

Specificity of *C. trachomatis* LCR. Sixteen organisms potentially found in clinical specimens plus human placenta DNA and the closely related *C. psittaci* and *C. pneumoniae* were tested by LCR to determine amplification specificity. None of the three LCR probe sets amplified any of the specificity panel DNA to detectable levels. The panel included *Lactobacillus plantarum*, *Haemophilus ducreyi*, *Fusobacterium mortiferum*, *Yersinia enterocolitica*, *Corynebacterium hoffmanii*, *Streptococcus faecalis*, *Pseudomonas diminuta*, *Proteus vulgaris*, and *Haemophilus influenzae* tested at 2.5 × 10^6 organisms per amplification. *Bacteroides fragilis*, *Candida albicans*, *Neisseria gonorrhoeae*, *Klebsiella pneumoniae*, *Gardnerella vaginalis*, *Staphylococcus epidermidis*, and *Escherichia coli* were all tested at 5 × 10^5 organisms per amplification reaction. *C. psittaci* and *C. pneumoniae* were tested at 200 organisms per amplification.

C. trachomatis DNA was included as a control in each set of amplification reactions and was amplified as expected.

With LCR an additional probe was not needed to confer additional specificity as was the case with PCR. Specificity is inherent in the probes in which even a 1-base mismatch near the junction of the probes inhibits ligation (15, 26).

LCR has proven to be a fast and sensitive amplification procedure. Amplification and detection by LCR were equivalent to those by PCR, and both methods may be able to detect organisms beyond clinically relevant levels. Detection of LCR product lends itself well to nonisotopic procedures through the use of haptens on either end of the probe pairs. When ligated together, the product would serve as a bridge between capture and detector antibodies. Without ligation, no bridge could be formed. This scheme could then be used in any of a variety of automated enzyme-linked immunoassay detection instruments.

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